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RECENT RESEARCH ON THE WESTERN CORN ROOTWORM

Genes, gene flow and adaptation of *Diabrotica virgifera virgifera*

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- Abstract**
- 1 *Diabrotica virgifera virgifera* has emerged as a major pest of cultivated maize, due to a combination of its high capacity to inflict economic damage, adaptability to pest management techniques and invasiveness.
 - 2 This review presents a survey of the current state of knowledge about the genetics of *D. v. virgifera*. In addition, the tools and resources currently available to *Diabrotica* geneticists are identified, as are areas where knowledge is lacking and research should be prioritized.
 - 3 A substantial amount of information has been published concerning the molecular phylogenetic relationships of *D. v. virgifera* to other chrysomelids.
 - 4 There is a growing literature focused on the population genetics and evolution of the species. Several adaptations to anthropogenic selection pressure have been studied, with resistance to synthetic insecticides providing some particularly well-characterized examples.
 - 5 A notable deficiency is a lack of studies directed toward the formal genetics of *D. v. virgifera*.

Keywords *Diabrotica virgifera virgifera*, evolution, genetics, insecticide resistance, western corn rootworm.

Introduction

The western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is arguably the single most important pest of field maize, *Zea mays* L. (Levine & Oloumi-Sadeghi, 1991; Sappington *et al.*, 2006), throughout most of the U.S. Corn Belt both in terms of crop losses and the use of synthetic insecticides. Managing corn rootworm populations to minimize the risk of economic loss is extremely difficult, in part because of its extraordinary capacity to evolve resistance both to chemical insecticides (Metcalf, 1986; Meinke *et al.*, 1998; Siegfried *et al.*, 2005; Parimi *et al.*, 2006) and cultural control practices such as crop rotation (Levine *et al.*, 2002). Recent management practices have relied extensively on neurotoxic and nonspecific synthetic insecticides that are directed against both larvae and adults. Corn rootworm management strategies that include prescriptive insecticide applications have not been widely

adopted (Gray & Steffey, 1995) placing increased pressure on the limited number of options available to growers.

The genetics of *D. v. virgifera* is a relatively new field of study. Nevertheless, rapid progress has been made in recent years and this trend seems likely to continue. This is due in part to the formation of the *Diabrotica* Genetics Consortium (Sappington *et al.*, 2006), a cooperative agreement between researchers with an interest in *Diabrotica* genetics that allows for the sharing of data and expertise and provides a forum for the development of more formal collaborations. An important aspect of the consortium is that its membership is not confined to dedicated geneticists but extends to specialists in ecology, behaviour and pest management who have a peripheral interest in the genetics of the species. This has fostered an atmosphere of cross-disciplinary collaboration to the benefit of all concerned. One consequence of the accelerating pace of *D. v. virgifera* genetics research is that a significant body of, as yet, unpublished information exists. To provide the fullest possible overview of the state of the field,

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we have indicated a number of important, unpublished developments that we are aware of in this review.

Much of the earliest work on *Diabrotica* genetics was concerned with phylogenetics and species identification. A natural development from this has been an interest in the population genetics of *D. v. virgifera* (and, to an extent, of congeneric species). There is also an active *Diabrotica* genomics community that is making progress in elucidating the molecular mechanisms involved in the species' adaptation to various selection pressures.

An important motivation for the study of *D. v. virgifera* is its importance as a major pest of agriculture. However, this species can also provide insights of much wider biological importance. It has shown a remarkable ability to adapt its metabolism and behaviour to a variety of pest management techniques. It is also a formidable biological invader, having first expanded its range to cover most of North America and, more recently, invaded Europe. Thus, *D. v. virgifera* is an excellent model species that can be used to deepen our understanding of adaptive evolution and biological invasions.

Available tools and resources

Cytoplasmic markers

Mitochondrial DNA and, more generally, DNA from cytoplasmic organelles possess a number of desirable properties (Hartl & Clark, 1997: 361). Cytoplasmic genomes are inherited maternally so that ancestry relationships are more easily inferred than from nuclear genetic material: cytoplasmic gene lineages correspond to individual female lineages whereas nuclear gene lineages are not as simple. Moreover, the genomes of cytoplasmic organelles do not normally recombine (Wallis, 1999), so each of them can be considered as a single locus (Dowling *et al.*, 1990). Phylogenetic or phylogeographic relationships are thus directly inferred from the similarity of cytoplasmic DNA sequences between taxa or individuals. Additional properties make mitochondrial DNA a good candidate for genetic analyses: It evolves relatively rapidly and thus shows a large amount of variability; it is a short molecule and thus is easy to study; and it is present in several copies in each cell and thus is easy to extract and conserve.

Several cytoplasmic molecular markers have been used to elucidate the phylogenetic relationships and population genetic diversity of *D. v. virgifera*, and the closely-related Mexican corn rootworm, *Diabrotica virgifera zea*. These include the mitochondrial cytochrome oxidase I (COI) and II (COII) genes, NADH dehydrogenase 4, 12S rRNA, and 16S rRNA (Simon *et al.*, 1994; Szalanski & Powers, 1996; Szalanski *et al.*, 2000; Clark *et al.*, 2001b; Eben & Espinosa de los Monteros, 2004; Gillespie *et al.*, 2004).

The diversity of the obligate intracellular bacterium *Wolbachia*, which occurs in some populations of the two *D. virgifera* subspecies has been assessed with DNA sequence data as well. *Wolbachia* is widespread in arthropods and can cause several sex-altering phenotypes (Stevens *et al.*, 2001). The most common phenotype is cytoplasmic incompatibility. *Wolbachia*-infected females can successfully mate with both in-

fect and uninfected males and produce infected offspring, whereas uninfected females are at a disadvantage because they can mate only with uninfected males. As a result, *Wolbachia* spreads into uninfected populations of insects, carrying with it the concomitant host mitochondrial genome. Thus, the spread of a single strain of *Wolbachia* in an existing naïve population, or the spread of an invasive insect species from a few founder individuals, infected with a single strain of *Wolbachia*, can be tracked by measuring mitochondrial haplotype diversity.

Wolbachia in *D. v. virgifera* and *D. v. zea* populations have been identified using several bacterial genes. The 16S rRNA gene was amplified using primers 21F and 994R and 99F and 1492R (O'Neill *et al.*, 1992; Giordano *et al.*, 1997). The *ftsZ* gene, whose product contributes to the septation of bacterial cells was amplified using primers *ftsZf1* and *ftsZr1* (Werren *et al.*, 1995; Giordano *et al.*, 1997). The *wsp* gene encoding the major surface protein of *Wolbachia* has been amplified using primers 81F and 691R (Braig *et al.*, 1998; Zhou *et al.*, 1998).

Allozymes

Allozymes are variant forms of enzymes with differing electrophoretic mobility due to amino acid substitutions (Hartl & Clark, 1997: 45–46). Allozyme variation has been used extensively to study genetic diversity in both model and nonmodel species from the 1960s onward. Recently, however, the study of allozymes has largely been overtaken by DNA-based methods because DNA is generally more stable (and therefore easier to work with) and more variable than enzymes. To our knowledge, the only survey of allozyme diversity in *D. v. virgifera* was carried out by Krafur (1999) who examined several chrysomelid species collected in Iowa. The proportion of polymorphic loci in *D. v. virgifera* (19.4%) was lower than for other *Diabrotica* species (77.7% and 59.0% in *Diabrotica barberi* and *Diabrotica undecimlineata howardi*, respectively). However, heterozygosity at those loci that were polymorphic was comparable to that observed in other diabrotic species.

Microsatellites

Microsatellites are short tandem repeats of DNA sequences, which are highly polymorphic, and evenly distributed and abundant in genomes, occupying several thousand loci in eukaryotes and up to one million loci in the human genome (Ellegren, 2004). Microsatellites can be scored easily using polymerase chain reaction (PCR) amplification followed by genotyping with an automated sequencer. For these reasons and because of their co-dominant mode of inheritance, microsatellite loci are generally the markers of choice for numerous applications in ecological genetics (Parker *et al.*, 1998; Goldstein & Schlötterer, 1999; Zhang & Hewitt, 2003; Lowe *et al.*, 2004) and are often employed in linkage mapping and quantitative trait loci studies (Primrose, 2003; Ellis & Burke, 2007). They are especially valuable for inferring levels of genetic variation and population structure among closely-related or recently diverged populations (Roderick, 1996; Haig, 1998; Donnelly & Townson, 2000; Kim & Sappington, 2005b, 2006). These markers can be used to estimate rates and patterns of gene flow, and to directly

identify immigrants in a population and their likely region of origin using population assignment analyses (Waser & Strobeck, 1998; Davies *et al.*, 1999; Paetkau *et al.*, 2004; Miller *et al.*, 2005; Nardi *et al.*, 2005; Kim *et al.*, 2006).

The introduction and continuing range expansion of *D. v. virgifera* in Europe, concern that *D. v. virgifera* may develop resistance to transgenic *Bt* maize, and the ongoing spread of a crop rotation-tolerant variant in North America have combined to put a premium on obtaining estimates of gene flow and understanding the population genetic structure of this insect (Sappington *et al.*, 2006). Several laboratories in the U.S.A. and Europe were interested in developing microsatellite markers for *D. v. virgifera*. One disadvantage of microsatellites as markers is that they are expensive and time-consuming to develop. They are usually species-specific, so they must be developed anew for each species (Zane *et al.*, 2002), although certain loci sometimes can be amplified across closely related species (Ellis & Burke, 2007). The *Diabrotica* Genetics Consortium was organized in 2004 in part to coordinate efforts in marker development (Sappington *et al.*, 2006). Kim and Sappington (2005a) tested 17 microsatellites from 54 that were initially isolated from an enriched genomic library, and found that nine of these will probably be useful in population genetics studies. The others showed signs of harbouring null alleles, caused by mutations in the flanking regions of the microsatellite loci that prevent PCR amplification (Callen *et al.*, 1993; Behura, 2006). A heterozygous individual with a null allele that is artifactually invisible is falsely genotyped as a homozygote at that locus. Null alleles thus bias the results in population genetics analyses and microsatellites that are known to have one should be avoided (Pemberton *et al.*, 1995; Liewlaksaneeyanawin *et al.*, 2004; Dakin & Avise, 2004). Approximately 59% of *D. v. virgifera* microsatellites have null alleles (Kim *et al.*, 2008a). Waits and Stolz (2007) have developed a number of microsatellites from a combined *D. v. zea* and *D. barberi* genomic library. The markers that amplify *D. v. zea* loci also will amplify in *D. v. virgifera*, as was the reciprocal case for previously developed *D. v. virgifera* markers (Kim & Sappington, 2005a). Microsatellites designed from sequences mined from a *D. v. virgifera* expressed sequence tag (EST) database were tested against conventionally derived microsatellites, and performed comparably in population genetics applications (Kim *et al.*, 2008a). Thus, EST data mining represents a less expensive alternative for future marker development, and one such marker has already seen service in population studies of *D. v. virgifera* (Miller *et al.*, 2005).

Because coordination of marker development occurred early for *D. v. virgifera*, the opportunity was seized upon to develop a standard core set of microsatellite markers to be recommended for use in future population genetics studies (Sappington *et al.*, 2006). Laboratories in the U.S.A. (USDA-ARS, Iowa, and USEPA, Ohio) and France (INRA, Sophia-Antipolis) evaluated 22 potential markers for five desirable characteristics: high polymorphism, readability and repeatability on different sequencing systems, lack of null alleles, selective neutrality and no linkage between loci. Six markers performed well under these strict criteria and are recommended as a core set to be used in all studies (Kim *et al.*, 2008b). Use of these markers among any others included in future studies

will facilitate comparisons of data generated by different laboratories, and will allow direct sharing of genotype data, which can save significant time and resources.

Amplified fragment length polymorphism

Another class of nuclear DNA marker that is being applied to studies of *D. v. virgifera* is amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995). Briefly, the AFLP technique involves digesting genomic DNA with a pair of restriction enzymes. Oligonucleotide adapters are then ligated to the sticky ends of the digested DNA. Because the sequences of the adapters are known, they can then be used as targets for PCR primers. There is considerable scope for 'tuning' AFLP protocols to suit organisms with different genome sizes and base compositions. In particular, the choice of restriction enzymes or the number of 'selective' bases at the 3' end of the PCR primers (extending beyond the adapters) can be varied to change the number of fragments that are amplified during a single PCR reaction. Typically, one of the PCR primers is radioactively or fluorescently labelled to allow visualization and sizing of the amplified fragments by traditional gel electrophoresis or using automated DNA sequencer technology. AFLP variation manifests itself as the presence or absence of a particular amplified DNA fragment. Thus, AFLP is a dominant/recessive marker system: heterozygous individuals cannot be easily distinguished from individuals that are homozygous for the 'positive' allele.

The key advantage of adopting an AFLP strategy is that it can provide hundreds of polymorphic markers quickly and cheaply. This makes it an attractive system for use in linkage mapping, genome scanning and other applications that demand large numbers of markers. The dominant nature of AFLPs may be a disadvantage in population studies, especially if no co-dominant markers are available to verify that populations conform to Hardy-Weinberg expectations.

The suitability of AFLPs for the study of chrysomelids has been demonstrated by their application to the Colorado potato beetle, *Lepinotarsa decemlineata*, both for linkage mapping (Hawthorne, 2001) and population genetics (Grapputo *et al.*, 2005). The AFLP protocol developed for *L. decemlineata* by Hawthorne (2001) also has been found suitable for studying variation in *D. v. virgifera* populations (Miller *et al.*, 2007). Although the application of AFLP to *D. v. virgifera* is in its infancy, it is likely that the technique will prove useful, particularly for the production of linkage maps.

Genomic resources

The genomes of living organisms contain many elements, including genes coding for proteins. The portion of genes expressed as mature mRNA, collectively known as the transcriptome, represents only a small percentage of the complete genome but contains much of the information of interest (Jongeneel, 2000). A snapshot of the transcriptome of a particular tissue or cell type can be obtained by producing a cDNA library and sequencing a sufficiently large number of individual clones to ensure that most of the information

present in the library has been extracted, resulting in a set of ESTs. Because there can be many genes from which more than one EST is derived, clustering of sequences through computational comparison of overlapping ends and the degree of sequence identity is employed to identify contiguous sequences or 'contigs'. Unique sequences or 'unigenes' are represented by sequences identified in only one clone.

At present, the only genomic resource to be reported in the peer-reviewed literature for *D. v. virgifer* is an EST database derived from larval gut tissue (Siegfried *et al.*, 2005). The cDNA library from mRNA extracted from dissected *D. v. virgifer* larval midguts contained 2.53×10^{10} primary clones. Sequencing of 2880 clones was conducted of which 1528 usable sequences were assembled into 190 contiguous sequences (contigs) and 501 unique sequences. The average length of readable sequences was 635 nucleotides. Each unique sequence was searched against the nonredundant GenBank database using the BLAST algorithm. Of the 691 unique sequences, 27% (187) did not return any significant ($E \leq 10^{-5}$) BLASTX matches. Of the remaining 504 sequences, 71% had best matches to insect sequences; specifically 42% to *Drosophila melanogaster* and 29% to other insects. Those sequences returning a significant BLASTX match were ascribed a putative biological process and molecular function (The Gene Ontology Consortium 2001; <http://www.geneontology.org>) based on the single 'best hit' match. Molecular functions correspond to activities that can be performed by individual gene products, whereas biological processes are accomplished by one or more ordered assemblies of molecular functions. Strikingly, 80% of the sequences predicted proteins with either catalytic activities (61.8%) or binding functions (18.8%). Correspondingly, 74% of sequences were predicted to encode proteins involved in either metabolism (64.5%) or transport (9.1%).

A preliminary estimate of the size of the *D. v. virgifer* genome by H. Robertson (University of Illinois) and S. Johnston (Texas A&M University) indicates that it is rather large, approximately 2.5 Gbp (Sappington *et al.*, 2006), making whole genome sequencing a daunting prospect. However, the rapid pace of improvements in the efficiency of genome sequencing and reductions in costs may make such an effort more attractive in the near future. EST databases will continue to be an important resource for identifying genes of interest. However, the EST databases must be expanded to include other tissues (e.g. nervous system, larval fat body) and additional sequencing of existing libraries should be conducted to ensure more complete transcriptome coverage. It is likely that the genomics resources available for studying *D. v. virgifer* will increase significantly in the near future. A substantial set of ESTs from adult head tissues has already been produced (H. Robertson, S. Ratcliffe, J. Thimmapuram, L. Lin & G. Gong, unpublished data; GenBank accessions EW761110–EW777362). In addition a cDNA microarray chip has been constructed using a combination of unique cDNAs from adult head and larval midgut tissues representing approximately 6600 genes (G. Robinson, University of Illinois; personal communication).

Laboratory colonies and strains

Although not a trivial undertaking, *D. v. virgifer* can be reared continuously in the laboratory (Jackson, 1986), and a number of colonies are maintained in the U.S.A. and Europe. The USDA-ARS North Central Agricultural Research Laboratory (NCARL) in Brookings, South Dakota maintains many colonies, including a line selected for nondiapause in the early 1970s (Branson, 1976). *Diabrotica v. virgifer* undergoes an egg diapause (Krysan, 1978; Meinke *et al.*, 2009). A chill period of 4 months is needed to synchronize hatch (Krysan *et al.*, 1984; Jackson, 1986), resulting in long generation times that are often inconvenient for research activities. Branson (1976) selected a laboratory population for short diapause-duration, creating a 'nondiapause' colony after nine generations. Six generations per year are possible with this line (Branson *et al.*, 1981), making it attractive for use by researchers. By 2003, this line had been in culture without outcrossing for about 190 generations (Kim *et al.*, 2007c), so loss or change of the genetic diversity of the colony has been a concern. Larvae from artificial infestations of nondiapause eggs have compared favourably with wild-type populations in field tests (Hibbard *et al.*, 1999), except when soil temperatures are low at time of infestation (Branson *et al.*, 1981). Kim *et al.* (2007c) used microsatellite markers (Kim & Sappington, 2005a, b; Miller *et al.*, 2005) to compare genetic variability in the nondiapause line at NCARL with that of other NCARL colonies and wild-type populations. The nondiapause colony has lost about 15–39% of its genetic diversity relative to wild-type populations, depending on the measure, but neutral genetic variation in the diapause colonies is similar to that of wild-type populations. If researchers desire a nondiapause colony for selection experiments, it is recommended that they introgress the nondiapause trait into a wild-type background to increase the amount of genetic variation available for selection, rather than working with the nondiapause colony directly (Kim *et al.*, 2007c).

Insecticide resistant strains of *D. v. virgifer* are currently maintained by NCARL. These strains have been characterized for resistance both to the cyclodiene insecticide, aldrin and the organophosphate insecticide, methyl parathion. Resistance characterization is based on dose–response assays for aldrin, diagnostic methyl parathion bioassays and on biochemical characterization of nonspecific esterase activity that is associated with methyl parathion resistance.

Patterns of neutral variation

Phylogenetics and phylogeography

Most published work using cytoplasmic markers in *D. v. virgifer* is related to molecular species determination and phylogenetic studies. PCR-restriction fragment length polymorphism (RFLP) on NADH dehydrogenase 4 (ND4) (Szalanski & Powers, 1996), the long-PCR amplicons 12S-N4 and CB2H-C1 (Szalanski *et al.*, 1999) and COI (Clark *et al.*, 2001a) were used to find diagnostic markers of *Diabrotica* species with various degrees of success. The ND4 locus could

be used to distinguish among *Diabrotica undecimpunctata*, *D. barberi* and *D. v. virgifera* (Szalanski & Powers, 1996) and variation in the COI gene provided a key to distinguish among 12 species of *Diabrotica* (Clark *et al.* 2001a). However, Szalanski *et al.* (1999) could not distinguish *D. v. zea* from *D. v. virgifera*, nor differentiate between *D. v. virgifera* subpopulations using mitochondrial PCR-RFLPs.

DNA sequences obtained from COI (Szalanski *et al.*, 2000; Clark *et al.*, 2001b; Gillespie *et al.*, 2003, 2004) and COII (Szalanski *et al.*, 2000) have proved useful to infer phylogenetic relationships between *Diabrotica* and other Luperini species. Sequence variation in the COI gene and the second internal transcribed spacer region ITS-2 was used to infer the phylogenetic relationships of thirteen *Diabrotica* species belonging to the *fucata* and *virgifera* species groups (Simon *et al.*, 1994; Clark *et al.*, 2001b). Eben & Espinosa de los Monteros (2004) reconstructed the phylogeny of 15 diabroticines, including *D. v. virgifera* and *D. v. zea*, in addition to several closely-related genera, using sequence data from mitochondrial COI, 12S rRNA, and 16S rRNA. Gillespie *et al.* (2003, 2004) used mitochondrial COI sequence data, along with that from nuclear 28S rRNA, to reconstruct phylogenies within the Luperini, including Diabroticina. They concluded that cucurbitacin feeding by rootworm adults does not reflect an ancestral host shift away from cucurbit host plants, but rather has evolved independently several times.

Although similar in many respects (Lance *et al.*, 1992; Spurgeon *et al.*, 2004), there are some ecological and physiological differences between *D. v. virgifera* and *D. v. zea* (Woodson & Chandler, 2000). Based on differences in elytral maculation patterns, the absence of pre-mating barriers to reproduction, unidirectional incompatibility and geographic distribution, Krysan *et al.* (1980) designated *D. v. virgifera* and *D. v. zea* as subspecies. This subspecies demarcation is useful because the latter superficially resembles *D. barberi* and some populations of *Diabrotica longicornis*, whereas populations of *D. longicornis* from New Mexico, Arizona and Mexico have black elytral markings similar to *D. v. virgifera*.

Both *D. v. virgifera* and *D. v. zea* are thought to have originated in Central America and invasively spread in the U.S.A. in relatively recent times (Smith, 1966; Branson & Krysan, 1981; Krysan & Smith, 1987). First collected in western Kansas in 1865 by Le Conte (Krysan & Smith, 1987), *D. v. virgifera* began to expand its range concomitantly with the spread and increase of continuous maize production in North America in the mid 20th Century (Chiang, 1973; Metcalf, 1983), and reached the east coast by approximately 1990. Between them, *D. v. virgifera* and *D. v. zea* are now distributed in the U.S.A. from Arizona and the Dakotas to New York and Virginia and into southern Mexico and southern Canada (Krysan & Smith, 1987). Early genetic studies of *D. v. virgifera* and *D. v. zea* populations showed little genetic diversity within and between the two subspecies (Krysan *et al.*, 1989; Szalanski *et al.*, 1999). This is in spite of the fact that some local populations show unique phenotypic characteristics such as pesticide resistance (Ball & Weekman, 1963; Meinke *et al.*, 1998), adaptation to crop-rotation (Stewart *et al.*, 1995; Levine & Oloumi-Sadeghi, 1996;

Levine *et al.*, 2002) and the presence or absence of infection with *Wolbachia* (Giordano *et al.*, 1997).

Giordano *et al.* (1997) tested whether the unidirectional incompatibility first determined by Krysan *et al.* (1980), between *D. v. virgifera* and *D. v. zea* was due to the presence of *Wolbachia* bacteria present in populations of *D. v. virgifera*. They found that full compatibility was restored when tetracycline-treated, and thus uninfected, *D. v. virgifera* males mated with *D. v. zea* females. A study of *D. v. virgifera* and *D. v. zea* populations throughout the U.S.A., showed that a single strain of *Wolbachia* (wDiavir) is present in *D. v. virgifera* beetles from northern Texas to New York, whereas populations in southeastern Arizona and populations of *D. v. zea* in Oklahoma and Texas are not infected. Moreover, populations of *D. v. virgifera* in the northern Mexican state of Durango, where a hybrid zone occurs between *D. v. virgifera* and *D. v. zea* (Krysan & Smith, 1987), were also found to be uninfected with *Wolbachia*.

Given the recent spread of *D. v. virgifera* in the Corn Belt and the distribution of *Wolbachia* infection, Giordano *et al.* (1997) proposed that the infected population of *D. v. virgifera* in the northern U.S.A. had probably undergone at least a cytoplasmic bottleneck and that as a result the genetic diversity of this very large population would be reduced with respect to its probable uninfected parental population in southeastern Arizona or Mexico. Preliminary mitochondrial DNA data from the COI and ATPase genes appear to support this hypothesis (R. Giordano, unpublished data).

Population structure, gene flow and movement

Just as cytoplasmic markers have been used to examine the evolutionary relationships between *D. v. virgifera* and other diabroticine species, nuclear markers, especially microsatellites, have proved useful for studying the relationships between populations of *D. v. virgifera*. All these studies use some of the six loci proposed for the standard core set (Kim *et al.*, 2008b). However, none has used the core set in its entirety, which has only recently been formulated.

Kim and Sappington (2005b) used seven loci to investigate the degree of geographical population genetic structuring in the U.S.A. They examined samples at a broad spatial scale ranging from Texas to New York. They found very little significant genetic differentiation between samples ranging through the Corn Belt to the east coast, although their sample from Illinois was significantly different from those from Pennsylvania and Delaware. A principal component analysis also indicated that the Illinois sample was slightly different from those in the rest of the Corn Belt.

Microsatellites also were used to study *D. v. virgifera* population structure at a finer geographical scale, mainly within Illinois (Miller *et al.*, 2006). The main objective of this study was to test whether the rotation tolerant variant form of *D. v. virgifera* is a genetically distinct population. Samples of adult *D. v. virgifera* were taken from paired maize and soybean fields in the region of Illinois where the variant was present. An additional sample was obtained from western Illinois, where the variant was absent at the time. To increase the number of wild-type samples, the Iowa and

Ohio data of Kim and Sappington (2005b) also were included in the analysis. In keeping with the results of Kim and Sappington (2005b), the study revealed no significant geographical population structure.

A second study that also focused on the problem of rotation tolerance (Miller *et al.*, 2007) compared samples of pupae from rotation tolerant populations in Illinois and wild-type populations in Iowa. A weak but statistically significant genetic differentiation was detected between the two areas using both microsatellites and AFLP markers. This result differs from those of Kim and Sappington (2005b) and Miller *et al.* (2006) in terms of statistical significance but is in keeping with the finding of Kim and Sappington (2005b) in that the Illinois population was slightly (albeit nonsignificantly) different from those in the rest of the Corn Belt. The slight discrepancy in statistical significance may indicate that there is a low level of general genetic differentiation between variant and wild-type *D. v. virgifer* that is detectable when pupae are sampled but not when adults are sampled. In both the Kim and Sappington (2005b) and Miller *et al.* (2006) studies, the samples of adults from Illinois may have been admixed samples of variants and wild-type, thereby obscuring the difference between the two types.

The use of microsatellites has not been confined to studying North American populations of *D. v. virgifer*. They have also been used to elucidate the routes by which the species has been introduced into various European countries (Meinke *et al.*, 2009). The description of the introduction routes of *D. v. virgifer* is important because they determine the genetic variability and thus the adaptability of the invasive populations in Europe. Particularly, the probability that insecticide resistance or rotation tolerance occur in Europe depends on the genetic characteristics of the source populations of invading *D. v. virgifer* and on the number of separate introductions from North America into Europe.

Studies of the introduction routes of *D. v. virgifer* into Europe have relied on an Approximate Bayesian Computation (ABC) framework (Beaumont *et al.*, 2002). The ABC approach allows the combination of genetic (microsatellite) and historical (years when European outbreaks of *D. v. virgifer* were first detected) data and quantitative comparisons of different putative introduction scenarios to be made. The ABC analysis considered a series of population triplets (two European populations plus North America). For any given triplet, the possible introduction scenarios were two independent introductions from North America, a single introduction from North America into Europe followed by an intra-European introduction and two independent intra-European introductions from a fourth unconsidered population, itself founded from North America. The overall introduction history was then reconstructed by combining the best models for each triplet. In the context of the triplet analyses, the fourth population could either be a known population that was not included in a given triplet or, in principle, a European population that was not sampled. Much of the power of the analysis derives from the loss of genetic diversity due to bottlenecks associated with new introductions. This being the case, it should be noted that independent introductions from North America could not be distinguished

from the special case of independent intra-European introductions from an unsampled European population that did not experience any loss of genetic diversity during its foundation (Meinke *et al.*, 2009).

The analysis of introduction routes (Miller *et al.*, 2005) made use of the microsatellites employed by Kim and Sappington (2005b) plus an additional locus, identified from EST data (Kim *et al.*, 2008a). The results of this study showed that there had been at least three direct introductions into Europe from North America: southeast Europe, Piedmont (northwest Italy) and Roissy (near Paris). There had also been two intra-European introductions from Roissy into Alsace (eastern France) and from southeast Europe into Friuli (northeast Italy).

Adaptive evolution

Novel control techniques are being developed and marketed for corn rootworm management. The two most recent and significant developments involve transgenic corn hybrids expressing insecticidal genes from *Bacillus thuringiensis* (*Bt*) and seed treatments employing neonicotinoid insecticides. Both technologies have the potential to drastically reduce the environmental and human health risks associated with conventional rootworm management practices (e.g. soil insecticides). However, the remarkable history of adaptation by *D. v. virgifer* to the selective pressures imposed by recent pest management practices necessitates the proactive implementation of management strategies designed to sustain these novel alternatives. Moreover, because of the invasive nature of this pest, proactive intervention for the purposes of mitigating invasions or minimizing the spread of resistance outbreaks are likely to be important aspects of future management decisions.

Cyclodiene resistance

Cyclodiene insecticides were commonly used as soil treatments for the control of both western and northern corn rootworms from the late 1940s to early 1960s. Benzene hexachloride (Muma *et al.*, 1949), aldrin, chlordane (Ball & Hill, 1953) and heptachlor (Ball & Roselle, 1954) were the recommended active ingredients for control of root feeding larvae during this period. Control failures with these compounds were first noted in Nebraska in 1959 (Roselle *et al.*, 1959), and further evaluations in 1960 (Roselle *et al.*, 1960) and 1961 (Roselle *et al.*, 1961) revealed the magnitude and rapid development of the resistance. During 1961, *D. v. virgifer* adults were collected from different fields in Nebraska and susceptibility to aldrin and heptachlor was determined by topical application (Ball & Weekman, 1962, 1963). Differences in susceptibility among field populations provided the first direct evidence of resistance evolution.

The development of cyclodiene resistance coincided with the rapid eastward range expansion of *D. v. virgifer*. By 1980 the distribution of the species covered most of the U.S. Corn Belt, including areas where cyclodienes were not widely used as soil insecticides (Metcalf, 1986; Meinke

et al., 2009) and has persisted in populations for many years after the use of these compounds was discontinued (Siegfried & Mullin, 1989). Parimi *et al.* (2006) reported the presence of high levels of resistance in both laboratory-reared and field-collected adult WCR based on topical bioassays with the cyclodiene insecticide, aldrin. Aldrin resistance apparently has remained consistently high among field populations over the four decades after resistance was first reported. These high resistance levels have persisted in spite of reduced selective pressures after the chemical class was banned in the U.S.A. in 1972. However, considerable variation in resistance levels among populations was detected with a general decline in resistance among Nebraska populations and consistently higher levels of resistance in more eastern populations. The general trend for higher resistance levels among populations where selection pressures are believed to have been lowest is puzzling. The use of broadcast applications of cyclodiene insecticides was generally confined to the western Corn Belt where resistance was first identified, and the higher resistance levels in eastern North America seem counter to the geographic gradient in selection intensity.

The only strain examined to exhibit complete susceptibility to aldrin was a nondiapause laboratory colony that was established from field collections in 1968 (Branson, 1976). This strain was derived from a field collection made in an area where resistance was reported to have been present at the time of collection (Metcalf, 1986). Because up to six generations of the nondiapause strain can be reared in the laboratory in a single year (Branson *et al.*, 1981), slight fitness disadvantages may have been manifested in the loss of resistance over a shorter period of time relative to field populations. It should also be noted that the nondiapause population has probably undergone a rather restrictive genetic bottleneck during selection for the nondiapause trait (Kim *et al.*, 2007c). Therefore, in selecting for a nondiapause trait, the genes conferring resistance could have been lost and the susceptibility of this strain may be unrelated to possible fitness disadvantages.

The mechanisms of cyclodiene resistance in *D. v. virgifera* remain uncertain. Comparisons of detoxification enzyme activities and *in vivo* and *in vitro* aldrin metabolism among resistant *D. v. virgifera* and the closely related but susceptible northern corn rootworm *D. barberi* revealed no consistent differences (Siegfried & Mullin, 1990). Recent research has suggested that target site insensitivity associated with a γ -aminobutyric acid (GABA) receptor-ionophore is a likely resistance mechanism (B. Siegfried, unpublished data). As a receptor for the major inhibitory neurotransmitter in insects, the GABA receptor is an important target for a number of insecticides including the cyclodienes (ffrench-Constant *et al.*, 2000; Ramond-Delpech *et al.*, 2005). Subsequent to a GABA-receptor subunit encoding a resistance-associated mutation (*Rdl*) being first isolated from a dieldrin resistant strain of *Drosophila melanogaster* (ffrench-Constant *et al.*, 1991), *Rdl*-like mutations have been found in several other insect orders (ffrench-Constant *et al.*, 2000). In the past, resistance to cyclodienes accounted for over 60% of reported cases of pesticide resistance in insects (Georghiou, 1969). However,

cyclodienes have been largely withdrawn from use and, consequently, the overall number of cyclodiene resistant species has been declining. Nevertheless, cyclodiene resistance remains an important model of target site-mediated resistance and has been used in a number of instances to glean information regarding the genetic architecture of resistance (ffrench-Constant, 2000).

In most cases studied, resistance appears to involve insensitivity of the GABA receptor, caused by a conserved point mutation that results in an amino acid substitution of an alanine either to serine or glycine within the second transmembrane domain (M2) (Hosie *et al.*, 1997). Significant progress has been made recently in two specific areas related to cyclodiene resistance in *D. v. virgifera*: (i) identification of an *Rdl* mutation associated with cyclodiene resistance and (ii) identification of significant variation in susceptibility to the cyclodiene insecticide aldrin among *D. v. virgifera* populations (B. Siegfried, unpublished data). Because of the rapid range expansion and persistence of cyclodiene resistance in *D. v. virgifera*, detectable variation in susceptibility and availability of molecular markers, cyclodiene resistance in this species represents a potentially important model for understanding the evolution and movement of target site-mediated resistance genes.

Organophosphate resistance

Organophosphate and carbamate insecticides were introduced after the failure of cyclodienes and successfully replaced these compounds as the predominant rootworm insecticides throughout the U.S. Corn Belt. Both organophosphates and carbamates are still used as soil insecticides against larvae and as foliar insecticides in adult management programs. Both soil insecticides and adult rootworm control were adopted as primary management tools where irrigated, continuous maize is planted over large acreages throughout the Platte River valley of central Nebraska. However, in some areas of Nebraska, aerially applied microencapsulated methyl parathion was used almost exclusively (Meinke *et al.*, 1997) over relatively large areas and in consecutive years.

Control failures of aerially applied methyl parathion were first reported in the early 1990s, and resistance to organophosphate and carbamate active ingredients was documented in rootworm adults from a number of Nebraska populations (Meinke *et al.*, 1998). The distribution of resistant rootworms was initially restricted to areas of the state where adult management had been practiced far more than 10 years, whereas areas relying on soil insecticides and crop rotation apparently remained susceptible. It also has been determined that rootworm larvae are resistant to a number of organophosphate and carbamate active ingredients, and that the same metabolic mechanisms are present across life stages (Miota *et al.*, 1998; Wright *et al.*, 2000). However, the spectrum of resistance is relatively narrow and does not appear to affect performance of commonly used soil insecticides.

Slight insensitivity of acetylcholinesterases to inhibition by methyl-paraoxon (the neurotoxic metabolite of methyl parathion) has been identified in resistant Phelps County

beetles (Miota *et al.*, 1998), and elevated cytochrome P450-based metabolism of carbaryl (Scharf *et al.*, 1999a) has been noted in populations from both York and Phelps Counties. However, synergism studies using the esterase inhibitor DEF (S,S,S-tributyl phosphorothioate) indicate a common involvement of esterases in both methyl parathion (Miota *et al.*, 1998) and carbaryl resistance (Scharf *et al.*, 1999b). Insecticide metabolism studies employing [14 C] ethyl parathion (Miota *et al.*, 1998) and [14 C] carbaryl (Scharf *et al.*, 1999a) showed increased formation of hydrolysis products and disappearance of parent insecticides. Relative to susceptible rootworm larvae, elevated esterase activity has also been noted in populations resistant to multiple acetylcholinesterase inhibiting insecticides (Wright *et al.*, 2000). Together, these findings emphasize the importance of hydrolytic metabolism as a cross-resistance conferring mechanism that occurs across life stages and over relatively large geographic areas.

A diagnostic bioassay involving exposure to a residue of methyl parathion on the inside of a glass vial was developed for quickly assessing the resistance status of field-collected rootworm populations (Zhou *et al.*, 2002). Based on the response curves of representative resistant and susceptible populations, a diagnostic concentration corresponding to the LC₉₉ of a standard susceptible colony was determined. This concentration was used to assess resistance by identifying the proportion of a given population that exceeds 1% survival at the LC₉₉ for a susceptible population. Based on sampling results over a 4-year period using the diagnostic concentration of methyl parathion, resistance exhibited significant expansion both in distribution and in intensity.

Initial sampling of rootworm susceptibility in 1996 indicated the presence of two distinct resistant areas based on the presence of susceptible populations that separate these two regions (Fig. 1). However, by 1998, significantly increased levels of tolerance were observed in the areas of York and Hamilton counties, and areas previously identified as being susceptible had become highly resistant (Adams County; Fig. 1). Although resistance appeared to be growing both in intensity and in geographic range, there were still populations of rootworms that remained susceptible to methyl parathion in close proximity to resistant populations. Furthermore, in areas where aerially applied methyl parathion no longer provided effective control of adult rootworms, growers adopted other management practices such as crop rotation and use of soil insecticides.

In representative resistant populations, esterase activity toward α - and β -naphtholic esters was approximately five- to six-fold higher than susceptible baselines (Miota *et al.*, 1998). Visualization of esterase isoenzymes from mass homogenates of rootworm abdomens on native polyacrylamide gels has consistently shown a similar trend (Zhou *et al.*, 2002). On these native gels, three esterase isoenzyme groups are typically visible, with one showing elevated activity in resistant populations (group 2). Interestingly, when individual abdomens are homogenized and separated on native polyacrylamide gels (i.e. one abdomen per lane), only the group 2 esterases of resistant individuals are visible. During the 1998 growing season, 26 *D. v. virgifera* populations of varying methyl parathion susceptibility (based on survival at a diag-

nostic concentration of methyl parathion) were evaluated to determine the proportion of individuals in these populations having elevated esterase activity by native PAGE. Elevated esterase activity-frequency was well correlated with survival on methyl parathion diagnostic concentrations ($r^2 = 0.898$) (Zhou *et al.*, 2002).

The development and spread of methyl parathion resistance provides an important tool in validating models of resistance evolution. This approach was recently used to validate a stochastic model of the evolution of resistance to adulticidal sprays of methyl parathion in *D. v. virgifera* populations in

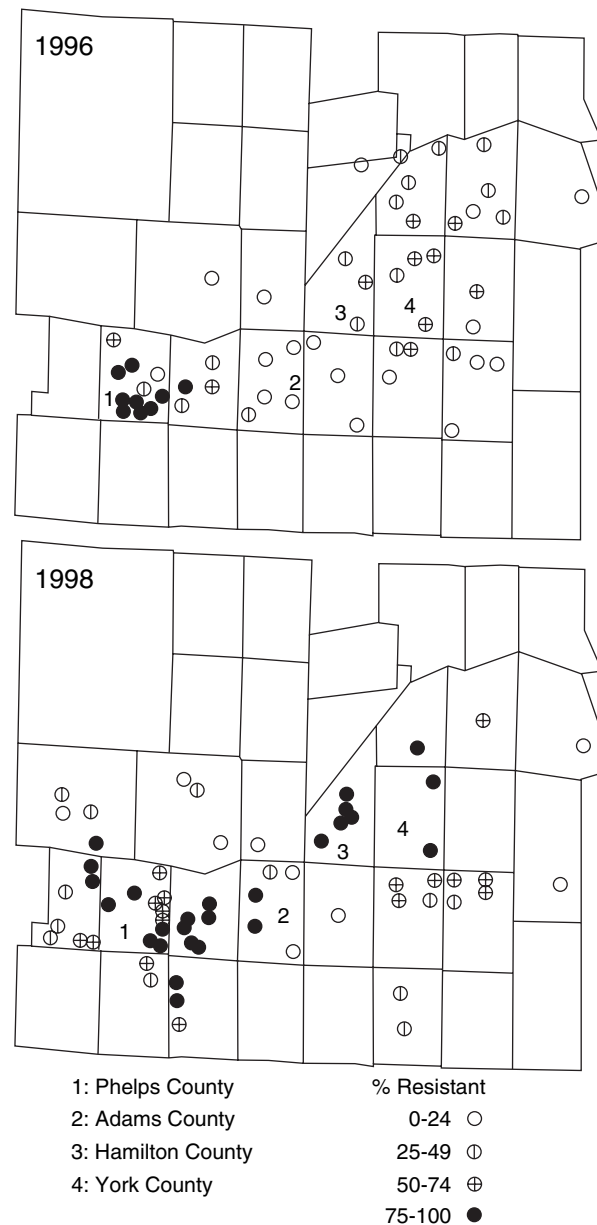


Figure 1 Distribution of methyl parathion resistant *Diabrotica virgifera virgifera* in central and southern Nebraska, based on the percentage of individuals surviving at a diagnostic concentration after 4 h (L. Meinke, unpublished data).

Nebraska (Caprio *et al.*, 2006). When resistance was examined as a genetic phenomenon, the rate of increase of the resistance allele depended almost entirely on genetic factors (LC_{50} values), the characteristics of the pesticide (residual activity), and the variance associated with emergence of adults. When resistance was measured as failure of methyl parathion to reduce populations below threshold levels (0.5 gravid females per plant), parameters that contributed to population growth rate (mortality and fecundity) were also important. These data suggest two important phases in resistance evolution in corn rootworms: a genetic phase associated with negative growth rates and rapid changes in resistance allele frequencies and a rebound phase associated with positive growth rates and near fixation of the resistance allele.

The precise molecular nature of resistance to organophosphate insecticides remains elusive. Specific cytochrome P450 gene fragments have been identified that appear to be over-expressed in resistant populations (Scharf *et al.*, 2001) but their involvement is as yet unconfirmed. With respect to enhanced hydrolytic metabolism, there is a clear indication that over-expressed esterases are associated with resistance. Enhanced hydrolytic metabolism of insecticide substrates also has been documented in resistant strains (Miota *et al.*, 1998; Scharf *et al.*, 1999a) but specific esterase genes have yet to be identified. However, a promising line of investigation is the use of cDNA microarrays to identify genes that are differentially expressed in previously identified organophosphate susceptible and resistant *D. v. virgifera* strains (B. Siegfried, unpublished data).

Adaptation to crop rotation

Crop rotation is an attractive alternative to insecticides for managing *D. v. virgifera*, at least in regions where it is economically and agronomically viable. Furthermore, crop rotation is a required element of the response to new outbreaks of the pest in Europe (European Commission, 2003). Crop rotation exploits the ovipositional fidelity of *D. v. virgifera* to maize (Hein & Tollefson, 1985), univoltine life cycle and limited larval host range (Gillette, 1912; Branson & Ortman, 1967, 1970; Chiang, 1973; Levine & Oloumi-Sadeghi, 1991; Clark & Hibbard, 2004). Maize grown in rotation with other crops (first year maize) remains free of *D. v. virgifera* larvae because the alternative crop did not present an attractive oviposition site the previous year.

Damage to first year maize by *D. v. virgifera* larvae was first observed in Ford County, Illinois in 1987 (Levine & Oloumi-Sadeghi, 1996). By 1995, maize producers across a large section of east central Illinois experienced severe root injury in their rotated maize fields. The problem has now spread throughout most of Illinois and into several nearby states (Levine *et al.*, 2002; Onstad *et al.*, 2003). The mechanism behind this phenomenon was not a prolonged diapause of *D. v. virgifera* eggs as had been observed for the northern corn rootworm *D. barberi* (Levine *et al.*, 1992). Instead, it was found to be due to high levels of oviposition into soybean fields that were rotated to maize the next year (Levine *et al.*, 2002). Occasional oviposition by WCR in soybean fields has been reported previously (Shaw *et al.*, 1978).

However, this was attributed to volunteer maize in soybean fields and this explanation was not viable as the primary factor responsible for the widespread damage to rotated maize fields in the mid-1990s.

An early laboratory study (Sammons *et al.*, 1997), suggested that *D. v. virgifera* from Indiana were attracted to soybean foliage. Adults collected from Indiana consumed more soybean leaf tissue than beetles obtained from Iowa or Nebraska. However, this finding was not supported by wind tunnel experiments that showed that beetles from 'problem' areas were not unusually attracted to soybeans (Spencer *et al.*, 1999).

Although progress recently has been made in determining why variant *D. v. virgifera* adults (particularly females) leave maize and disperse to adjacent soybean fields, a defined mechanism remains elusive. Populations of adults from both problem and nonproblem areas will feed on soybean foliage but neither appears to derive any nutritional benefit from doing so (Mabry & Spencer, 2003; Mabry *et al.*, 2004). Maize phenology has been shown to affect the amount of soybean foliage consumed by *D. v. virgifera* adults. Greater levels of soybean tissue were eaten when mature reproductive stage maize was present in contrast to younger vegetative stage maize (O'Neal *et al.*, 2002). However, this behaviour was common to adults collected from Illinois (where damage to first year maize was common) and from Nebraska and Michigan (where it was not). A subsequent investigation, using laboratory olfactometer chamber assays, found that *D. v. virgifera* adults moved to chambers containing soybean foliage more readily when corn foliage in other chambers began to senesce (O'Neal *et al.*, 2004). These results suggest that maize phenology rather than an adaptive change on the part of the beetle might explain the problem (O'Neal *et al.*, 2004). By contrast, large-scale field experiments in which maize and soybean phenology were manipulated by planting date have showed that, although variant populations respond to maturing maize by ovipositing in soybean fields, wild-type populations do not (Pierce & Gray, 2006a). It seems probable that oviposition into soybean fields (and thus damage to first year maize) is due to a reduced fidelity to maize rather than attraction to soybeans (Mabry & Spencer, 2003), which may be stimulated by advancing maize phenology (Pierce & Gray, 2006a). Additional research in commercial maize and soybean fields has further elucidated the dispersal characteristics, oviposition patterns, and survivorship of variant WCR in east central Illinois (Pierce & Gray, 2006b, 2007). Significant differences in locomotor activity between populations from problem and nonproblem areas also have been observed (Knolhoff *et al.*, 2006).

Although a genetic basis for the variant behaviour has not been demonstrated, it seems highly likely. Computer simulation studies by Onstad *et al.* (2001) have shown that the selection pressure imposed by the level of crop rotation typical of east-central Illinois would favour an adaptive allele that confers reduced specificity to maize. Further simulation studies by Onstad *et al.* (2003) have shown that the growth of the area in which first year maize is at risk can be explained by the dispersal (aided by storms) of a rotation-adapted (i.e. genetically-determined) population. Consequently, there have been attempts to identify genetic

markers associated with the variant phenomenon. Miller *et al.* (2006) analyzed the variation at eight microsatellite loci and could not identify any general genetic differentiation between adult WCR collected in soybeans and those obtained from maize in areas categorized as variant or non-variant. This implies that the rotation tolerant variant is probably not a reproductively isolated strain and that any molecular markers associated with the behaviour will be confined to the immediate genomic region of the gene or genes involved (Miller *et al.*, 2006). Subsequent to this study, Miller *et al.* (2007) attempted to identify AFLPs associated with the variant. This latter study made use of a more targeted sampling strategy than the former. Samples were collected as pupae from first year maize in east central Illinois and from continuous maize in central Iowa. Thus, the Illinois samples were guaranteed to be the offspring of females that had oviposited into soybean (i.e. variant females), whereas the Iowa samples could safely be assumed to be wild-type, given their geographical location. An AFLP marker associated with a gene involved in the variant behaviour was expected to show an elevated level of divergence between Illinois and Iowa, beyond that expected for neutral genetic loci. The distribution of divergence assuming selective neutrality, and thus the probability that a given marker was neutral, was determined by computer simulations (Beaumont & Nichols, 1996). Of 253 AFLP markers that were analyzed, only one showed evidence of being associated with the variant/wild-type difference. However, the level of differentiation between the two types at this locus was modest and the marker cannot be used to discriminate between them. This may be because the marker is only loosely linked to a gene involved in the variant behaviour. The failure of this study to identify a marker strongly linked to a 'variant gene' may well be a consequence of the large size of the *D. v. virgifera* genome.

The future of *Diabrotica* genetics: needs and opportunities

Although remarkable progress has been made in understanding the evolution, population genetics and genomics of *D. v. virgifera* there are still a number of key areas where knowledge is lacking. A particularly notable deficiency is the absence of any studies of the formal genetics of the species. It would be very useful to construct a linkage map for *D. v. virgifera*, which would facilitate quantitative trait loci studies of insecticide resistance (including resistance to *Bt* toxins), diapause, and other traits of interest. The microsatellites developed so far, especially the core set, will be useful in population genetics studies for many years to come and will undoubtedly be an important resource for the construction of linkage maps. However, the time and expense of developing microsatellites means that there will probably never be enough of them to adequately saturate a linkage map. AFLPs are used extensively for linkage mapping in other organisms, and they can be of value in population genetics work as well, but they are not always straightforward to work with and can be laboratory-specific. Their sometimes

idiosyncratic characteristics can make transfer of linkage maps between laboratories, for example, problematic. Thus, there is a need to develop additional kinds of genetic markers for *D. v. virgifera* that are abundant, simple to use, and robust. Such markers could include single nucleotide polymorphisms identified from EST databases and other sequencing projects.

As well as additional nuclear markers, more variable cytoplasmic markers would be useful to better resolve the evolutionary relationships between *D. v. virgifera* and *D. v. zeae*. Likely sources of highly variable cytoplasmic markers include the mitochondrial A + T rich control region and transposon associated polymorphisms in *Wolbachia*.

The development of a core set of microsatellites (Kim *et al.*, 2008b) illustrates the benefits of sharing reference DNA samples between institutions to synchronize allele calls. To date, arrangements for sharing material have been made on a case-by-case basis between the researchers involved. A more formal depository of *Diabrotica* DNA samples would be advantageous, especially to scientists who are new to the field. In principle, such a depository could house not only reference samples, but also material from individuals or populations with interesting phenotypes or other characteristics. Consideration should be given to a suitable venue for a DNA depository and the resources needed to ensure the secure storage and timely distribution of material. The *Diabrotica* Genetics Consortium (Sappington *et al.*, 2006) could provide a forum for discussions on the establishment of a DNA depository.

Most of our understanding of the genetics and evolution of *D. v. virgifera* (and indeed most aspects of the species' biology) comes from studies of North American populations. In comparison, rather little is known about European populations. In particular, it is unclear to what extent the adaptations that have allowed *D. v. virgifera* to overcome insecticide treatments and crop rotation are present in Europe. Determining this would help to inform management strategies for combating this invasive pest. In addition, such information may shed light on the location of the source population (or populations) in North America that founded the European populations. This is because although selectively neutral markers (i.e. microsatellites) are spatially homogenous over a large part of North America, adaptive traits, such as resistance to insecticides and to crop rotation, often exhibit appreciable spatial heterogeneity.

Identifying the source of the European introductions may also shed light on why there appears to have been a sudden burst of transatlantic introductions during the last two decades. This phenomenon poses a number of questions. For example, have populations of *D. v. virgifera* close to transatlantic airports only recently reached sufficient densities that the entry of beetles into aircraft is probable? Alternatively, have recent adaptations or environmental changes increased the propensity of *D. v. virgifera* to enter aircraft?

The most clear and pressing research need concerning the development of behavioural adaptation to crop rotation is a method for identifying individual insects as variant or wild-type. This could be either a refined behavioural assay or a

molecular marker. Although no such test exists at present, the results of both behavioural studies and searches for molecular markers suggest that either approach may yet prove fruitful. By contrast to the behavioural variation, markers associated with resistance to chemical insecticides by *D. v. virgifera* as well as spatial heterogeneity in the distribution of resistance levels among geographically distinct populations is becoming well established. This makes *D. v. virgifera* an excellent model to further our understanding of the evolution of insecticide resistance.

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